

A tumor-suppressing mechanism in *Drosophila* involving cell competition and the Hippo pathway

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Mutant larvae for the *Drosophila* gene *lethal giant larva* (*lgl*) develop neoplastic tumors in imaginal discs. However, *lgl* mutant clones do not form tumors when surrounded by wild-type tissue, suggesting the existence of a tumor-suppressing mechanism. We have investigated the tumorigenic potential of *lgl* mutant cells by generating wing compartments that are entirely mutant for *lgl* and also inducing clones of various genetic combinations of *lgl*[−] cells. We find that *lgl*[−] compartments can grow indefinitely but *lgl*[−] clones are eliminated by cell competition. *lgl* mutant cells may form tumors if they acquire constitutive activity of the Ras pathway (*lgl*[−] *UAS-ras*^{V12}), which confers proliferation advantage through inhibition of the Hippo pathway. Yet, the majority of *lgl*[−] *UAS-ras*^{V12} clones are eliminated in spite of their high proliferation rate. The formation of a tumor requires in addition the formation of a microenvironment that allows mutant cells to evade cell competition.

Drosophila imaginal discs | Hippo pathway | *lgl*

The formation of tumors normally occurs after a transformation of groups of cells that acquire a set of properties allowing them to proliferate in excess and to colonize normal tissues, superseding nontumor cells. In *Drosophila* there are a number of mutations known to cause excessive growth leading to the production of tumors. Among these mutations, there is the group of tumor suppressor genes *scribble* (*scrib*), *disc large* (*dlg*), and *lethal giant larvae* gene (*lgl*), whose function is necessary for normal cell polarity and asymmetric cell divisions (reviews in refs. 1–3). In mutant larvae for *scrib* and *lgl* the neuroblasts and imaginal cells develop massive neoplastic tumors that eventually kill the larvae. These tumors exhibit many of the properties of human tumors, including loss of tissue architecture and alterations of cell shape. Moreover, the human homologs of these genes are also associated with the formation of diverse types of cancers (4, 5).

These studies have identified genetic defects that may lead to tumor formation but are not informative about how tumors appear and progress within normal tissue. From this perspective, it is of interest to consider the behavior of cells mutant for *scrib* or *lgl*. Although mutant homozygous larvae for these genes develop extensive tumors in imaginal discs, clones of mutant cells surrounded by wild-type tissue do not produce tumors (6–9). Furthermore, Brumby and Richardson (6) and Igaki et al. (8) have shown that *scrib* tumorous cells are eliminated by JNK-dependent apoptosis. It appears that the potential of *scrib* and *lgl* mutant cells to form tumors depends on the cellular context: if they are surrounded by “like” cells they develop tumors, but if surrounded by normal cells they do not. This suggests the existence of a tissue-specific mechanism that recognizes individual features of cells and proceeds to the elimination of undesirable cells. This behavior resembles the phenomenon of cell competition (10–12); a compartment-specific short-range interaction between cells with different division rates that leads to JNK-mediated apoptosis of the slower dividing cells. A similar kind of interaction may also function to eliminate abnormal or malignant cells that may arise in development.

The observations that constitutive expression of the Ras pathway (6, 9) rescues the lethality of *lgl* or *scrib* clones and causes tumorous growth indicate that under certain conditions the tumor-suppressing mechanism can be evaded.

To address this issue we have analyzed the growth of discs and compartments mutant for *lgl* and also the behavior of clones of *lgl* mutant cells of various genetic combinations developing in normal (*lgl*/+) background. We find that *lgl* mutant clones are normally eliminated by a process akin to cell competition, but constitutive Ras activity (*lgl*[−] *ras*^{V12}) confers on the clones the potential to survive and generate tumors. *lgl*[−] *ras*^{V12} clones acquire high growth rate through down-regulation of the Hippo (Hpo) pathway, but in spite of their growth advantage many of these clones are also eliminated. Our results indicate that clones of *lgl* mutant cells developing in normal tissue can form a tumor when (i) the Hpo pathway is inhibited or down-regulated, which confers *lgl*[−] cells a high proliferation rate, and (ii) the groups of fast proliferating cells are able to merge together to generate a microenvironment that allows the group to overcome cell competition and to continue growing.

Results and Discussion

Entire *lgl*[−] Discs or Compartments Can Grow Indefinitely, but Isolated *lgl*[−] Cells Are Eliminated by JNK-Mediated Apoptosis. As reported long ago (13), *lgl* mutant larvae are unable to pupate and remain a long time in the culture medium to finally die as gigantic larvae when they are 12–13 d old. The CNS and the imaginal discs develop into extensive tumors that reach very large size (14), (Fig. S1C). We have studied the growth dynamics of the discs of mutant larvae (see Fig. S1 legend for details) and found that they grow actively as long as the larva is alive. Thus a principal feature of *lgl* mutant discs is that, unlike the wild type, they continue growing past the normal body and tissue size. We have reached a similar conclusion after studying the growth of posterior compartments that are entirely mutant for *lgl*. In larvae of genotype *lgl*[−] *FRT40A/M(2)24F FRT40A; UAS-Flp hh-Gal4* (see *Materials and Methods*) most or all of the posterior (P) compartment becomes homozygous for *lgl*, whereas the anterior (A) compartment remain *lgl*⁺ and serves as control. We find that whereas the control *lgl*⁺ anterior compartment stops growth once it has reached final size, the *lgl*[−] compartment continues growing and reaches a large size (Fig. S1 D–F). This can be shown by the comparison of the P/A size ratios in larvae of different ages. The posterior *lgl*[−] compartment is initially somewhat smaller than a wild type, the P/A size ratio is 0.43 ($n = 18$) in 144-h larvae, whereas the normal value is 0.65 (15). However, by 168 h, the P/A ratio is 0.84 ($n = 15$) and in 216-h larvae, it is 1.25 ($n = 10$). These observations indicate that *lgl* mutant cells proliferate indefinitely, as they do not respond to the general mechanism that arrests growth when the final stereotyped size of compartments (15) has

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some cases the whole clone becomes apoptotic (Fig. 3 E–F'). This suggests that the apoptosis observed in *lgl⁻ ras^{V12}* cells may be a general feature of tumorous cells growing in a normal tissue.

Tumorous *lgl⁻* Clones Are Eliminated by a Mechanism Akin to Cell Competition Irrespective of Their Cell Division Rate. Because the viability of the *lgl* mutant tissue appears to be normal, the elimination of *lgl* mutant cells when they are in the same population with *lgl⁺* cells suggests that it is due to cell competition. This is based on several observations: (i) the apoptotic cells are always inside the clones, indicating that only *lgl⁻* cells are being eliminated; (ii) the cells in apoptosis appear preferentially at the clone borders (Fig. 1 B, B', and E and Figs. 3 and 4), suggesting that it is caused by short-range interactions with *lgl⁺* cells located in the vicinity; (iii) the *lgl⁻* cells are eliminated by JNK-mediated apoptosis, a typical feature of cell competition (6, 8, 12); and (iv) the elimination of *lgl* mutant cells is compartment specific; it does not occur across compartment borders, as indicated by our experiment in which we generate a posterior compartment made exclusively of *lgl* mutant cells (Fig. S1). The close proximity of *lgl⁻* and *lgl⁺* cells at the A/P border does not result in apoptosis of *lgl⁻* cells.

The one difference between the cell elimination process we demonstrate here and classical cell competition is that the latter has been associated with slow division rate (10, 12). Our experiments indicate that *lgl⁻* cells proliferate approximately at the same rate as normal cells, and most importantly, that *lgl⁻ ras^{V12}* and *lgl⁻ UAS-Yki* cells proliferate more rapidly than *lgl⁺* cells and yet they are frequently eliminated.

In our view, cell competition can be defined in a more general way as a close-range interaction between two types of viable cells that leads to apoptosis of one type. This would include the classical example of slow dividing cells (10, 12) and also other similar interactions. Milan et al. (24) reported that clones of cells overexpressing *spalt* (*sal*) survive in the normal *sal* domain but are eliminated by apoptosis outside the domain. It appears that cells outside the *sal* domain recognize those overexpressing *sal* as “different” and drive them to apoptosis. Similarly, ventral or

dorsal disc cells were eliminated if they appear in the inappropriate compartment. Comparable observations have been made for other changes of identity (25, see review in ref. 26). These observations suggest the existence of an intrinsic mechanism to remove cells that respond abnormally to developmental cues (see ref. 27).

We propose that a similar process plays a role in the case of clones of mutant *lgl⁻* cells growing in *lgl⁺* discs. The *lgl⁻* clones are identified as being developmentally different through their interactions with neighbor *lgl⁺* cells. This leads to JNK induction and subsequently apoptosis in the mutant cells. It is a forceful mechanism able to remove fast proliferating cells like *lgl⁻ ras^{V12}* and *lgl⁻ UAS-Yki* cells. Along a similar line of thought, Igaki et al. (8), have proposed the existence of a mechanism to remove oncogenic cells on the basis of the endocytic activation, via Eiger, of the apoptotic JNK pathway.

***lgl ras^{V12}* Cells Need to Form a Microenvironment to Evade Cell Competition and Form a Tumor.** Finally, we have examined the ability of *lgl⁻ ras^{V12}* cells to form tumors. We consider that a disc contains a tumor when the *lgl⁻ ras^{V12}* cells occupy at least 50% of the disc and it also shows outgrowths and gross morphological alterations associated with *lgl⁻ ras^{V12}* cells.

As mentioned above, *lgl ras^{V12}* clones were induced by Flp-induced mitotic recombination under heat shock control (see *Materials and Methods*). Surprisingly, there is a large difference regarding the frequency of tumors in the 7- and 15-min HS experiments. Whereas in the 15-min experiment, 90% of the discs ($n = 91$) contain extensive tumors (Fig. 3D), only 8% of the discs ($n = 100$) develop tumors in the 7-min HS experiment.

The low number of tumors in the 7-min experiment can be explained by the results above, indicating that about half of the *lgl⁻ ras^{V12}* clones in the 7-min experiment are eliminated by apoptosis. To check this idea we have compared the frequency of *lgl⁻ ras^{V12}* tumors in *dronc⁺* and *dronc⁻* background. The results are the following: in the 7-min experiment tumor frequency in *dronc⁺* background after 96 h is 8%, whereas in *dronc⁻* ($n = 65$) it is 33%. Thus the conclusion is that only a small subset of *lgl⁻ ras^{V12}* clones is able to develop tumors and that apoptosis is playing a major tumor-suppressing role. Interestingly, a similar comparison in *dronc⁺* and *dronc⁻* discs in the 15-min HS experiment yield close values of tumor frequency of 91 and 100%, respectively (see below).

The 10-fold difference in tumor frequency between the 7- and 15-min experiments indicates that the higher clone density after 15 min of HS facilitates the formation of tumors. This idea in turn suggests that many tumors may result from merging of individual clones. This is supported by the examination of discs from the 15-min HS experiment, like the one in Fig. 2 C and C'; it contains several relatively small clones some of which are adjacent and will probably merge into individual patches to form a tumor. Other clones are likely to disappear as they contain many apoptotic cells.

The comparison of patch density in discs fixed 72 and 96 h after HS also suggests a merging process: in the 15-min HS experiment patch frequency per disc decreases from 12.3 ($n = 20$) in the 72-h discs to 5.1 ($n = 18$) in the 96-h discs. In contrast, in the 7-min HS experiment there is no significant difference in patch frequency in 72-h (1.9, $n = 25$) and 96-h discs (2.2, $n = 18$). These results indicate that the higher clone density in the 15-min HS experiment facilitates clone fusion, visualized by the diminution of patch frequency.

The idea that clone merging facilitates tumor formation is also supported by an experiment in which we discriminated the compartmental origin (anterior or posterior) of the *lgl⁻ ras^{V12}* clones using *engrailed* (*en*) expression as a marker. Of a sample of 29 discs containing massive tumors covering most of the disc, 26 contained anterior and posterior cells (Fig. 5 B and D), whereas 3 were composed exclusively of anterior cells. Even the rare cases of tumors in the 7-min experiment contain cells from anterior

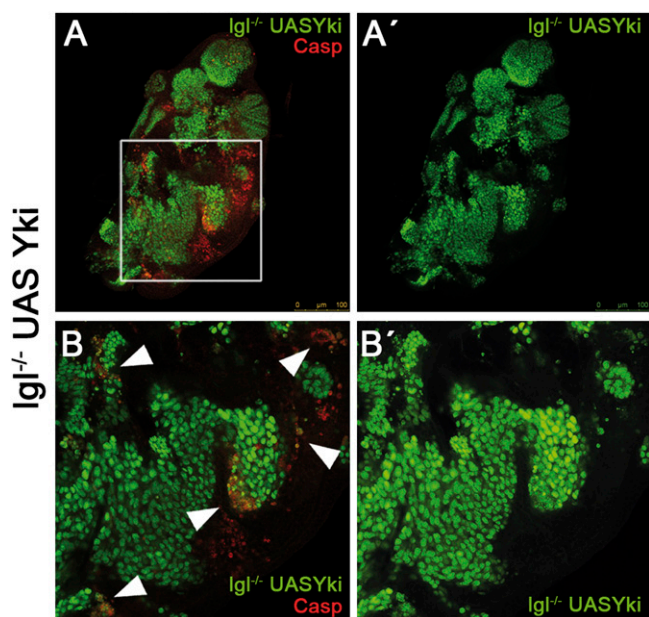


Fig. 4. Behavior of *lgl⁻ UAS-Yki* clones. (A and A') Wing disc almost filled with *lgl⁻ UAS-Yki* clones. Note the areas of apoptosis (red) close to the borders of the tumors. (B and B'). High magnification of a region of the disc in A. Note (arrowheads) the incidence of apoptosis in *lgl⁻ UAS-Yki* cells located in the tumor borders or in clones that are isolated.

and posterior origin (Fig. 5 *B* and *B'*). Thus, the great majority or all of the tumors are of mixed origin.

In our view, the formation of a tumor by *lgt⁻ ras^{V12}* cells requires the generation of a microenvironment that prevents the elimination of the tumor cells. We have identified two factors that contribute to this microenvironment. One is an increase in proliferation rate, which in the case of *lgt⁻ ras^{V12}* cells is achieved through down-regulation of the Hpo pathway. However, although it facilitates the confrontation with *lgt⁺* cells, alone is not normally sufficient, as we find that fast-dividing *lgt⁻ ras^{V12}* or *lgt⁻ UAS-Yki* cells are often outcompeted. A similar process appears to occur in *scrib⁻ ras^{V12}* clones, which are outcompeted (Fig. 3 E and F) even though they also show nuclear localization of Yki (Fig. S5 E–E'').

The second factor is clone merging. Because the interactions between lgl^+ and lgl^- acts only at short range (see Figs. 1, 3, and 4), the fusion of individual clones helps to generate an internal environment within the patch that is not accessible to cell competition. Although $lgl^- ras^{V12}$ cells in the border of the patch (Fig. 3D) can be eliminated, those in the center will be protected and because of their high proliferation rate will form large masses of overgrowing tissue. Our observation that most tumors are of mixed anterior and posterior lineages (Fig. 5) supports this view strongly. The fact that the inhibition of apoptosis in the 15-min experiment does not affect tumor frequency also supports this view. The high density of clones facilitates merging and functions as a tumor-promoting mechanism.

In conclusion, we envisage two major steps in the process of tumor formation in *Drosophila*: (i) the transformation of a normal cell into a tumor cell and (ii) the ability of the tumor cells to generate a microenvironment to confront the cell competition mechanism designed to eliminate them.

The first step may be caused by different genetic defects, including *lgl*, *scrib*, *dlg*, and possibly mutations in other tumor-suppressing genes. In the case of the *lgl* mutations, we believe that a critical feature is the property of *lgl* mutant tissue of not responding to the size control process: even though *lgl* mutant cells proliferate at a normal rate or perhaps even slightly lower, *lgl* mutant discs or compartments can grow indefinitely. This feature provides a tumorous property, but alone would not be tumorigenic in a normal growing disc.

The fulfillment of the second step is facilitated by the acquisition by the tumor cells of a high division rate, through the inhibition of the Hpo pathway or other causes. However, a high division rate appears not to be sufficient to escape cell competition. It also requires, at least in the case of *lgl⁻ ras^{V12}* and probably *scrib⁻ ras^{V12}* cells, a process of merging of a sufficient number of cells. Whether the cell merging process is an absolute requirement is an open question. It is conceivable that a very large difference in division rate between tumor and nontumor cells might be sufficient for individual clones to generate the necessary microenvironment to escape cell competition.

We wish to point out that in humans and other species tumors normally appear in the form of groups of malignant cells that are mixed with normal ones. The progression of the tumor requires that the malignant cells compete successfully with the normal surrounding ones. It is possible that in normal life the appearance of tumor cells may be a frequent occurrence, but they are normally eliminated by a process similar to that described here. Only after acquisition of some additional property, higher division rate and/or ability to generate a protective environment, they can successfully confront the tumor suppressing mechanism.

Materials and Methods

Mutant Stocks. The fly stocks used were obtained from the Bloomington Stock Center except where indicated. We used two different *Igl* alleles, *Iglrd* (a gift of Peter Bryant, University of California, Irvine, CA) and *Igl^{IM}*, an allele that appeared fortuitously in our laboratory. We have also used the chromosome *Df(2L)net62*, a physical deletion of *Igl* (28). The *Iglrd* allele has been characterized as a null (29). We consider *Igl^{IM}* as a null, on the basis of staining with anti-Lgl antibody and its interactions with the *Iglrd* allele and the deletion *Df(2L)net62*. In the experiments we used the *Iglrd* and *Igl^{IM}* alleles as convenient and refer to them as *Igl⁻*. The *scrib¹* mutation is described in ref. 6. The *dronc¹²⁹* allele (30) was also used to induce *Igl* mutant clones in a background resistant to apoptosis.

Generating *Igl* and *scrib* Mutant Clones. To induce *Igl*[−] clones by the MARCM system we used the stock *yw hsFlp122 tub-Gal4 UAS-GFP; tubGal80 FRT40A/Cyo*. Larvae were subjected to a 15- or 7-min heat pulse at 37 °C, 48–72 h after egg laying (AEL). We also used several UAS stocks to generate different genetic combinations of *Igl* mutant clones: *UAS-puc1TM6B*, *UAS-yki1TM6B* (a gift from Ken Irvine, Rutgers University, NJ), *UAS-Ras^{V12}/TM6B* and *puc^{E69}(puc-lacZ)/TM6B*.

To generate *scrib⁻ ras^{V12}* clones we used the *scrib¹ FRT82B* and the *Gal80 FRT82B* stocks with a protocol similar to that used to induce *Igl⁻ ras^{V12}* clones. In this case the heat shock time was 20 min.

To induce *lgl* mutant clones at the same time as control twin clones, we used the *ywhsFlp; ub-GFP FRT40A* stock and the *lgl⁴* allele.

To make entire compartments Ig^L we used the stock yw *hsFlp122; M(2L)24F ub-GFP FRT40A/Cyo; hh-Flp/TM6B* and the Ig^4 allele. All crosses were performed at 25 °C.

The size of the clones was measured with Wright Cell Imaging Facility ImageJ software.

Immunohistochemistry. Imaginal discs were dissected in PBS and fixed in paraformaldehyde 4% for 30 min at room temperature. The washing and the dilution of the antibodies were performed in PBT. The antibodies used were rabbit anti-cleaved Casp3, 1:50 (Cell Signaling Technology), mouse anti- β Gal, 1:50 (Hibridoma Bank), rabbit anti-Yki, 1:200 (a gift of K. Irvine), rabbit anti-PH3, 1–400 (Cell Signaling Technology), rabbit anti-P35, 1:5,000.

(Stratagene), guinea pig anti-dMyc, 1:1,000. Secondary antibodies were purchased from Invitrogen. For the BrdU detection we used the BrdU labeling kit from Sigma.

Samples were mounted in Vectashield and imaged in Leica TCS SPE and Zeiss LSM510 confocal microscopes.

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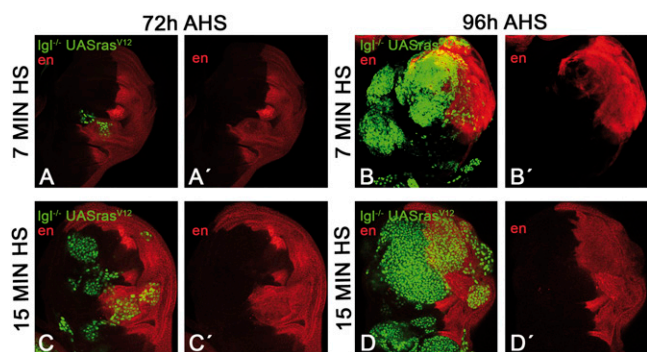


Fig. 5. Merging of *Igf1⁺ ras^{V12}* clones in tumors. The expression of *engrailed* (*en*, in red) marks the anterior or posterior compartment origin of the clones. The *Top* row shows two discs from the 7-min HS experiment. The disc in *A* and *A'* is the typical case fixed 72 h after clone initiation, in which there are very few clones and these are small. The disc in *B* and *B'* was fixed after 96 h and represents the unusual case in which the disc develops a tumor after 7 min of HS. Note that the tumor contains cells of anterior and posterior origin and therefore must have been formed by at least two clones. The *Bottom* row shows typical cases from the 15-min HS experiment. The 72-h AHS disc (*C* and *C'*) contains several clones, some anterior and some posterior. Note the closeness of some clones, suggesting they are about to merge. The 96-h AHS disc (*D* and *D'*) has a large tumor that contains cells of anterior and posterior origin.

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